



## Chemical composition of the sclerotized black coral skeleton (Coelenterata: Antipatharia): a comparison of two species

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The chemical composition of the skeletons of two black coral species, *Antipathes fiordensis* from New Zealand and *A. salix* from the Caribbean, was examined to compare elemental and protein composition, and the chitin content both between species and between colonial tip and base. Of all elements surveyed, iodine and bromine were dominant in the skeleton. In *A. fiordensis* these halogens constituted 2-3% of the skeletal weight, with different proportions occurring in tip and base. In contrast, the halogen content of *A. salix* constituted about 5% of the skeletal weight and consisted primarily of iodine in both tip and base. The chitin content of *A. salix* skeleton was about twice that of *A. fiordensis* based on <sup>13</sup>C NMR analysis, but the glucosamine content was more significantly different between tip to base of *A. fiordensis* than between species, and suggests that the chitin content of both is about 10-15% of the skeletal weight. The protein content of the two species was similar, constituting about 50% of the skeletal weight and varying by as much as 10% between the branch tip and base. Tip-to-base protein differences varied inversely with the chitin content. The skeletal proteins were rich in histidine, which constituted ca 11-14% of the amino acids. Both species also contained a tyrosine-rich fraction associated with chitin. The most distinctive difference between the skeletal proteins of the two species was the disparity in the levels and types of diphenol as shown by <sup>13</sup>C NMR and chemical analyses. In *A. fiordensis*, the skeleton contained about four and 12 times as much hydrolyzable DOPA in tip and base, respectively, compared to *A. salix*. Small amounts of 3,4-dihydroxybenzaldehyde were also present in *A. fiordensis* skeleton, but none was found in *A. salix*.

**Key words:** Antipatharia; Black coral skeleton; Antipathin; Diphenols; Sclerotization; Chitin; *Antipathes fiordensis*; *Antipathes salix*.

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### Introduction

Antipatharians are arborescent colonial coelenterates commonly called black corals. This

group of about 150 species is named after the color of the skeleton and was used as amulets and pharmancons in ancient times (Hickson, 1924). While the skeleton continues to have commercial value as jewelry, only recently have aspects of histology and structural organization (Goldberg and Taylor, 1989a,b; Goldberg, 1991) as well as skeletal biomechanics and architecture (Kim *et al.*, 1992) been documented. All

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species examined thus far have a substantial chitin component, ranging from about 6 to 18% of the skeletal weight (Goldberg, 1978, 1991; Ellis *et al.*, 1980). However, the dominant fraction of the skeleton is protein, which has an unusually high histidine content, ranging from 12 to 18% of the amino acids (Roche *et al.*, 1963; Goldberg, 1976, 1978). The term "antipathin" was coined for this distinctive material (Roche and Eysseric-Lafon, 1951) prior to the discovery of the chitin component. We continue to use the term antipathin in this paper to refer only to the protein content of the antipatharian skeleton.

The most thorough examination of antipatharin chemistry to date has been of *Antipathes fiordensis* described by Grange (1990), a shallow water species endemic to the Fiordland district of New Zealand. In general, its chemical properties are similar to those of hardened insect exoskeleton (Holl *et al.*, 1992). As in insect cuticle, many of the properties of antipathin suggest protein stabilization by quinone tanning or sclerotization. Tanned proteinaceous structures of marine invertebrates typically contain DOPA (Waite, 1990), as well as elevated levels of other aromatic amino acids, especially tyrosine, which is often halogenated (Roche *et al.*, 1963). The resulting skeletons are brown to black and virtually inert to the effects of acids or bases, oxidizing or reducing agents, and proteolytic enzymes (Brown, 1950). Antipathins examined thus far appear to possess all of these characteristics (Goldberg, 1976). Although some of the chemical changes that occur during maturation have been described (Goldberg, 1978), the identity of potential tanning agent precursors has been reported only in *A. fiordensis* (Holl *et al.*, 1992). Recently, Kim *et al.* (1992) reported that *A. fiordensis* differs significantly from a Caribbean black coral species, *A. salix*, described by Pourtales (1880), in both chitin fibril architecture and mechanical properties. This paper further examines the chemical composition of the skeletons of these two species with emphasis on the identification of potential protein cross-linking agents that may account for their different properties.

## Materials and Methods

Colonies of *A. fiordensis* were collected by Scuba at depths of 10–20 m from Doubtful Sound, New Zealand; *A. salix* was collected at depths of 30–40 m from Cay Sal Bank, Bahamas. After removal of tissue with jets of fresh water, the skeletons were dried and ground in a Wiley mill with a 1 mm screen. For elemental analysis, contact with metal was

avoided by grinding the skeletons in liquid nitrogen using an agate mortar and pestle. Four types of chemical analyses were applied to both the youngest branchlets (<0.3 mm in diameter), and the older, more basal parts of the colonies (>5 mm diameter): solid state  $^{13}\text{C}$  NMR for organic composition, HPLC for catechols, X-ray spectroscopy for trace elements, and amino acid analysis of antipathin hydrolysates.

### Solid-state NMR analysis

Samples from two colonies of each species were analyzed by cross-polarization, magic angle spinning (CPMAS)  $^{13}\text{C}$  NMR, analyzing multiple tips and bases separately to form composite spectra from each region of each species. Spectra were obtained at room temperature at 50.3 MHz. Spinning sidebands were suppressed using the TOSS pulse sequence (Dixon, 1982). Cross-polarization transfers were performed at 38 kHz with proton dipolar decoupling at 80 kHz. Rotors with 1 g sample capacities were made from ceramic (zirconia) barrels fitted with plastic (Kel-F) end caps and were supported at both ends by air-pumped journal bearings. These experiments used 400 mg samples positioned in the center of the rotor. Magic-angle spinning was at 3.205 kHz. For additional details of methodology, see Holl *et al.* (1992).

### Analysis of skeletal hydrolysates

Total protein, chitin and amino acid composition were determined after hydrolysis in 4 M methanesulfonic acid at 105°C for 20 hr (Simpson *et al.*, 1976) using a Joel 5AH amino acid analyzer with a ninhydrin-based detection system. Tips were analyzed from eight *A. fiordensis* colonies, with corresponding base samples from six of them ( $N = 14$ ). Material for *A. salix* was more limited; tips and bases from each of three colonies ( $N = 6$ ) were analyzed. Protein was estimated by integration of the total ninhydrin reactivity. Total chitin was estimated from the glucosamine content, correcting for hydrolytic deacetylation. Triplicate skeletal samples (tips only from three colonies; 2–3 mm diameter, 80–250 mg) of each species were hydrolyzed in 1 M KOH (105°C, 24 hr). The fibrous residue, representing a crude chitin preparation, was rinsed to neutral pH with distilled water, dried at 70°C in porcelain plates, cooled and desiccated over silica gel; the residual weight was then compared with the original sample weight. The chitinous residue was then rehydrated overnight in distilled water, mechanically separated into thin layers, and extracted with 1 M  $\text{H}_2\text{SO}_4$  for 3 hr at 25°C (cf. Brine and Austin, 1981). The acid-treated residue was

rinsed, dried and weighed as above. Amino acids and total protein remaining after treatment of skeleton with KOH and H<sub>2</sub>SO<sub>4</sub> were determined after *in vacuo* hydrolysis using 6 M HCl (analyses in triplicate). Duplicate 1 g samples of each species were also hydrolyzed in HCl for extraction of melanin according to the method of Hori *et al.* (1984).

#### *o*-Diphenol analysis

Free or weakly bound catechols were extracted from the skeletal powder of tip and base pairs from two colonies of each species by macerating samples in ground glass tissue grinders in ice-cold 1.2 M HCl with 5 mM ascorbic acid followed by hot 1.2 M HCl (100°C, 10 min). Peptidyl DOPA was liberated by heating powder from skeletal tips and bases in 6 M HCl with 5% phenol for 24 hr at 100°C. Free catechols were then adsorbed, recovered from alumina, and quantitated by reversed-phase high-performance liquid chromatography with electrochemical detection (HPLC-EC) (Morgan *et al.*, 1987; Holl *et al.*, 1992).

#### Elemental analysis

Elemental composition of skeletal samples was determined by proton-induced X-ray emission (PIXIE) spectroscopy (Elemental Analysis Corp., Tallahassee, FL). Two tip and base pairs from each species were ground to a powder using a 200–400 mesh screen and compressed to a 100-mg thick target (>18 mg/cm<sup>2</sup>). The pellets were placed in a helium-filled target chamber in tandem with a 1.7 × 10<sup>6</sup> v General Ionex accelerator and a Si(Li) detector. Data reduction was completed using proprietary software that normalized detected X-ray intensities against those measured from pure standards for each element (cf. Reuter *et al.*, 1975).

## Results

#### Solid-state NMR analysis

The <sup>13</sup>C NMR spectra from basal regions of *A. salix* and *A. fiordensis* (Fig. 1) showed similarities in chemical composition. Prominent peaks showing differences in concentration of aromatic, diphenolic, and chitin carbons are labeled in Fig. 2. Because the peaks were acquired using cross-polarization from protons, absolute numbers for the quantities of carbon-containing species cannot be given without relaxation measurements (Fukamizo *et al.*, 1986), but relative comparisons can be made between carbons of the same type. The region of 70–155 ppm indicated differences in both the diphenolic and chitin content between skeletal base and tip of both species (Fig. 2). Comparing

## BLACK CORAL BASE

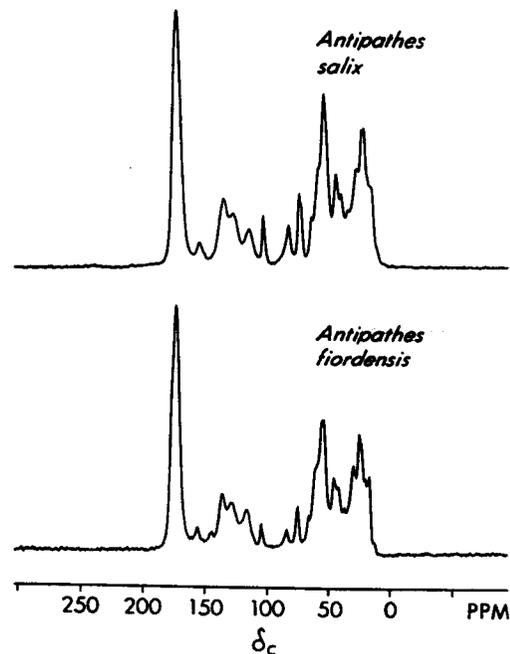


Fig. 1. Comparison of the 50.3 MHz CPMAS <sup>13</sup>C NMR spectra of the skeletal bases of the black corals *Antipathes salix* and *A. fiordensis*. Note congruent peaks in these composite spectra.

the species as a whole, approximately twice as much chitin was found in *A. salix* compared to *A. fiordensis*, but substantially more material with a diphenolic signature occurred in *A. fiordensis* than in *A. salix*, as indicated by a comparison of the resonance intensities at 145 ppm. This diphenolic region was particularly elevated in the base compared to the branch tips of *A. fiordensis*. The region between 125 and 140 ppm, which is primarily due to aromatic carbons in the amino acid side-chains of proteins, indicated the presence of slightly more protein in the base than in the tips of *A. salix*, but the reverse for *A. fiordensis*. In addition, more chitin was present relative to the aromatic carbon content in the younger, apical portions of *A. salix* than in the older, basal regions.

#### Analysis of skeletal hydrolysates

A comparison of protein content and amino acid composition revealed only small differences between species and between tip and base of the same species (Fig. 3). Three amino acids accounted for 60–62% of those detected. Glycine accounted for 33.3% of the residues in the tips of both species and in the base of *A. salix*; the glycine content of *A. fiordensis* increased slightly to 36.2%. Alanine accounted for 14.6 ± 0.5% of the amino acids, changing

## COMPARISON OF BLACK CORALS

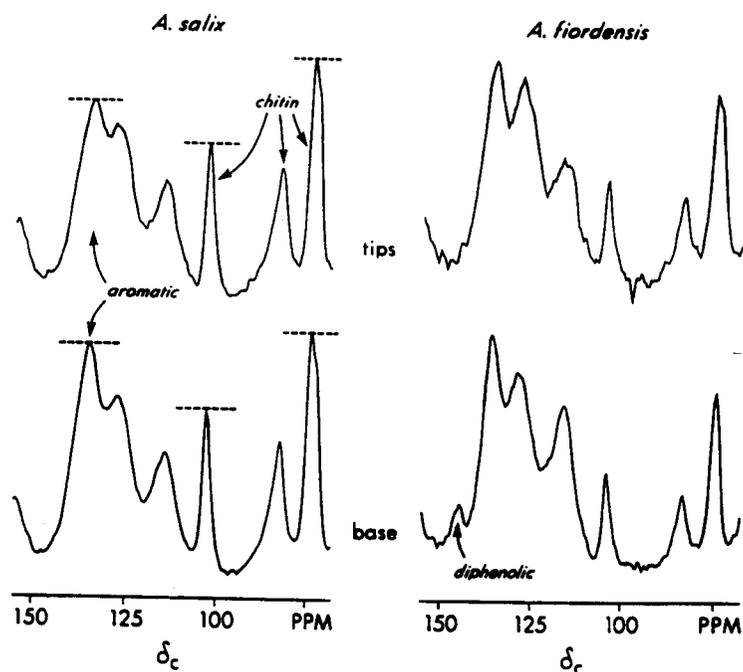


Fig. 2. Detail of the carbon chemical shift range  $\delta_c$  70–155 ppm comparing the tip and base regions of *A. salix* and *A. fiordensis*. Composite spectra show respective peaks representing chitin, aromatic compounds, and diphenols. Note the much greater diphenol peak in *A. fiordensis* and the relatively greater chitin peaks in *A. salix*. Using the dashed lines as a guide, more chitin can be seen relative to the aromatic peaks in the tips than in the base of *A. salix*. In *A. fiordensis* these differences are less apparent because of the lower chitin content.

little between regions or species. Histidine increased slightly from 11.3 to 12.7 mol.% in *A. salix* tip and base, respectively, while in *A. fiordensis* it decreased from 14.0% in the tip to 11.7% in the base. In *A. fiordensis*, the proportion of protein decreased from 55.4% (SE = 1.02) of the skeletal weight in the tip to 45.5% (SE = 1.06) in the base. The glucosamine (chitin) content of this species exhibited a concomitant increase from 10.4% (SE = 0.78) of the skeletal weight in the tip to 14.5% (SE = 1.06) in the base. In *A. salix*, the protein content was 51.3% (SE = 0.35) in the tips, but, in contrast to *A. fiordensis*, was slightly higher in the base (56.0%; SE = 1.16). Skeletal chitin in *A. salix* was 14.7% (SE = 0.69) in the tips and 13.4% (SE = 0.92) in the base. A statistical summary of protein and chitin by species and region is given in Table 1. There is general agreement between relative levels of protein determined by NMR and chemical methods. However, the small difference in the relative levels of chitin between species calculated by the glucosamine content does not correspond to the 2-fold difference seen by NMR. The basis of this disparity has not been resolved.

The skeletons of both species responded similarly to alkaline hydrolysis, losing approxi-

mately 89% of the protein content. In *A. salix*, the crude chitin residue was yellow, whereas in *A. fiordensis* it was brown. Further treatment of the *A. salix* residue with dilute  $H_2SO_4$  removed the yellow color, but with a relatively small weight loss (0.2%). For *A. fiordensis*, acid treatment changed color from brown to pink, with an additional weight loss of 0.9%, presumably representing a greater relative amount of chitin-bound protein in this species. Further hydrolysis of the crude chitin residue with 6M HCl revealed that some protein had resisted both the alkali and dilute acid treatments. Alanine, glycine, aspartic acid, histidine, and tyrosine were prominent in the hydrolysates of the chitin residue (Table 2). The greatest species difference among these amino acids was noted for histidine, with greater amounts of this amino acid remaining in *A. fiordensis* chitin. However, the total histidine content of the crude chitin preparation was less than that of untreated skeleton (Fig. 3). Tyrosine was clearly the dominant residual amino acid in the crude chitin residue of both species, in contrast to the relatively low percentage of this amino acid in untreated skeleton.

Melanin pigment was not detected in *A. fiordensis* (<5  $\mu g/g$  skeleton), whereas traces

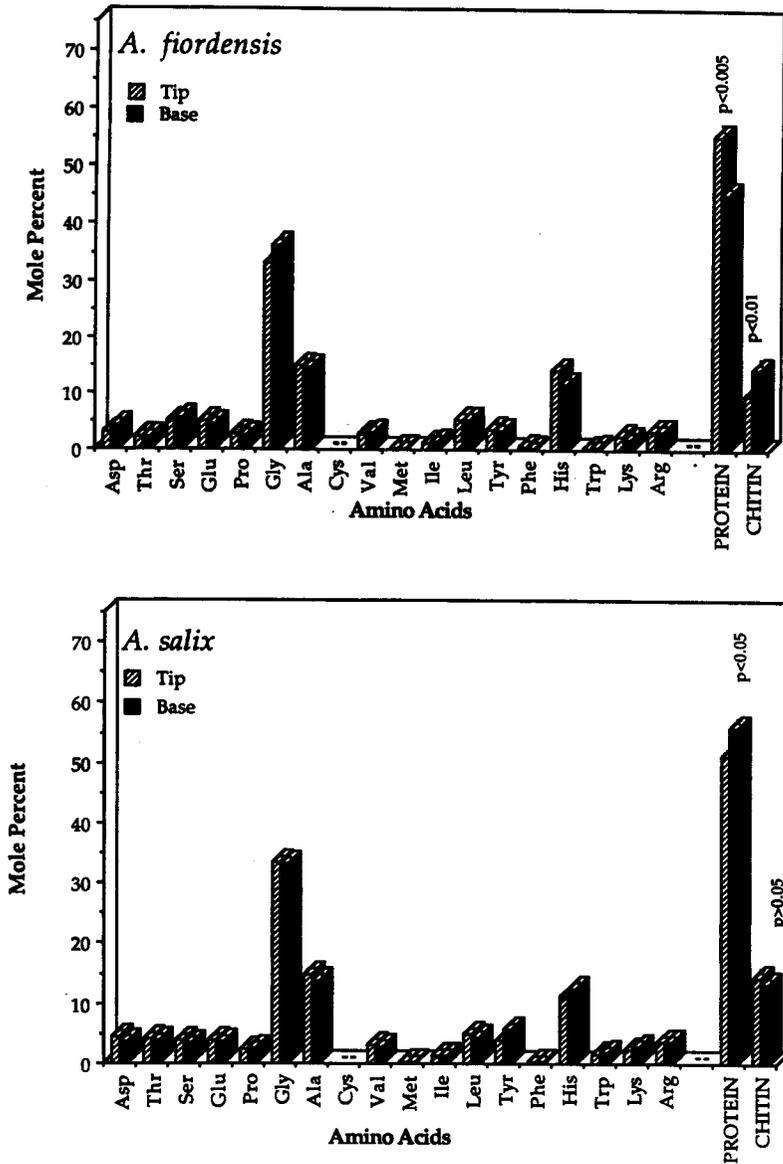


Fig. 3. Amino acid composition of tip and base antipaths from *A. fiordensis* and *A. salix* expressed in moles %. Chitin and protein are expressed as % organic weight. Bars = standard error; statistics are from Table 1.

Table 1. Differences in protein and chitin values were analyzed using Kruskal-Wallis one-way ANOVA corrected for ties

Skeletal component	Average rank		$\chi^2$	P
	Tip	Base		
<b>I. Protein</b>				
<i>A. fiordensis</i>	10.5	3.5	9.75	0.002
<i>A. salix</i>	2.0	5.0	3.97	0.050
Species difference in tip			6.00	0.025
Species difference in base			5.45	0.020
<b>II. Chitin</b>				
<i>A. fiordensis</i>	4.9	10.9	7.06	0.008
<i>A. salix</i>	4.7	2.3	2.33	0.127
Species difference in tip			5.04	0.050
Species difference in base			1.68	0.200

Table 2. Amino acid content of crude chitin residues from black coral skeletal tips\*

Amino acid	<i>A. salix</i>	<i>A. fiordensis</i>
Asp	9.2 (0.5)	9.5 (0.9)
Glu	4.4 (0.3)	4.7 (0.2)
Ser	5.6 (0.5)	4.9 (0.3)
Gly	10.8 (0.7)	11.3 (1.2)
His	7.5 (1.2)	10.4 (1.8)
Arg	3.4 (0.2)	3.2 (0.8)
Thr	6.8 (0.8)	5.1 (0.04)
Ala	12.7 (1.0)	11.2 (1.6)
Pro	2.9 (0.06)	3.3 (0.3)
Tyr	14.8 (1.7)	12.5 (0.8)
Val	6.7 (0.3)	5.6 (0.3)
Met	2.0 (0.6)	1.3 (0.2)
Cys	0.0	0.0
Ile	4.0 (0.04)	3.2 (0.3)
Leu	4.9 (0.4)	4.2 (0.3)
Phe	1.5 (0.2)	1.7 (0.2)
Lys	2.8 (0.1)	2.6 (0.3)

\*6 M HCl hydrolysis of chitin residue prepared by alkaline hydrolysis followed by extraction of skeletal samples with dilute H<sub>2</sub>SO<sub>4</sub>. Expressed in moles % (standard error); N = 3.

(6–10 µg/g) of acid-insoluble black material were recovered from *A. salix* skeleton. The melanin was analyzed from the mid-colony region of the skeleton, and we did not attempt to distinguish maturational differences between tip and base.

#### *o*-Diphenol analysis

3,4-Dihydroxyphenylalanine (DOPA) was detected by HPLC-EC in the 6 M HCl hydrolysates of both coral species (Table 2). However, approximately four times as much DOPA was

released from *A. fiordensis* tips and 12 times more was recovered from the base compared to *A. salix*. The amounts of DOPA occurring in *A. salix* were not only much smaller, they also did not differ significantly between tip and base. Because most of the DOPA was released only by 6 M HCl hydrolysis, it probably occurs as peptidyl DOPA and is an integral part of the antipatharian structural proteins.

Cold or hot dilute HCl (1.2 M) extracts of the skeletal powder suggest that levels of free or unbound DOPA in both species is small compared to the amount released by 6 M HCl hydrolysis (Table 3). However, the amount of DOPA released by cold or hot dilute acid was several times larger in *A. fiordensis* skeleton compared to *A. salix*. Significant amounts of 3,4-dihydroxybenzaldehyde (DOBAL) were also released by cold or hot acid extraction of *A. fiordensis* skeleton (Table 3 see also, Holl *et al.*, 1992), whereas none was detected in *A. salix* skeletal samples.

#### Elemental analysis

Elemental analysis of the skeleton in these two species showed both similarities and differences in composition. Of the 22 elements detected in the skeleton, sodium, chlorine, bromine and iodine were present in relatively large concentrations (Fig. 4). Sodium and chlorine levels vary together and are probably due to seawater. Bromine levels increased basally in both species, with higher relative concentrations in *A. fiordensis* compared to *A. salix*. The dominance of bromine relative

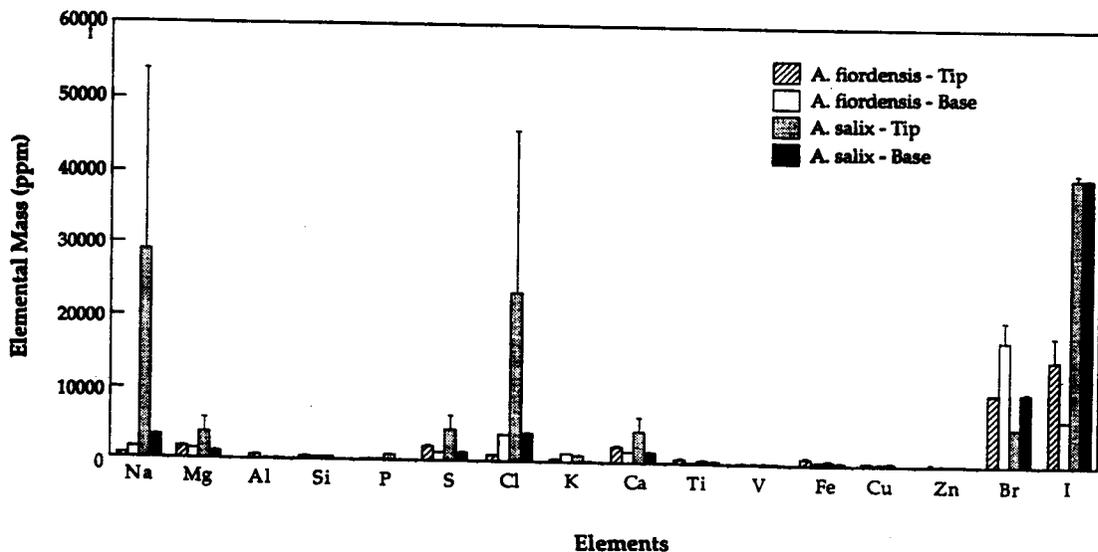


Fig. 4. Analysis of elements in tip and base of *A. fiordensis* and *A. salix* skeletons. Of the 22 elements detected Cr, Mn, Ni, Sr, As and Pb, are not shown due to irregular presence and/or low concentrations (< 100 ppm). Note the levels of iodine and bromine in comparison to other elements. Error bars show the range between two data sets.

Table 3. Concentration of *o*-diphenols (pmol./mg) from acidic extracts of *Antipathes fiordensis* and *A. salix* skeletal tips and bases\*

Sample region extract	<i>A. fiordensis</i>		<i>A. salix</i>	
	DOPA	DOBAL	DOPA	DOBAL
<b>Tips</b>				
6 M HCl	15,397 (2,000)	—	3,866 (700)	—
1.2 M HCl—cold	9 (8)	72 (30)	3 (2)	ND
—hot	55 (20)	120 (16)	8†	ND
<b>Bases</b>				
6 m HCl	40,948 (2,000)	—	3,460 (452)	—
1.2 M HCl—cold	36 (2)	60 (70)	4 (6)	ND
—hot	322 (204)	140 (126)	8 (2)	ND

\*Samples of skeletal powder were heated in 6 M HCl with 5% phenol at 100°C for 24 hr or were ground in ice-cold 1.2 M HCl with 5 mM ascorbate in a ground glass tissue grinder. The residue obtained after centrifugation was heated in HCl-ascorbate for 10 min at 100°C. The supernatants from each hydrolysate or extract were analyzed by HPLC-EC (see Materials and Methods for details). Means followed by (range); *N* = 2, ND = not detected.

†One sample.

to iodine in the base of the former species is noteworthy because such inversions have not been reported previously (Roche *et al.*, 1963; Goldberg, 1978). Iodine levels were higher in the tip than the base of both species, but the iodine concentrations were much higher in *A. salix* and constituted about 4% of the skeletal weight. The total iodine and bromine content of this species was about 5% of the skeletal weight.

## Discussion

### Biochemistry and mechanical properties

Physical and mechanical properties of black coral skeletons are likely to reflect the nature, relative abundance and architecture of their structural polymers, as well as the type and number of intra- and intermolecular bonds that stabilize them. The skeleton of the Caribbean black coral, *A. salix*, is stiffer, darker, harder, denser and more hydrophobic than that of the New Zealand species (Kim *et al.*, 1992). The chitin component functions as a fibrillar reinforcing agent that often serves to stiffen skeletal materials at low volume fractions (Wainwright *et al.*, 1976). Thus, higher chitin content has been associated with more pliant insect cuticles, whereas lower amounts are often found in stiffer cuticle (Hillerton, 1984). The chitin content of *A. salix* by NMR was about twice that of *A. fiordensis*. Using glucosamine levels to estimate chitin, Kim *et al.* (1992) found that *A. salix* tips contained  $\approx 29\%$  more chitin than *A. fiordensis*, essentially the same results as in this study. However, we found an insignificant difference in the chitin levels from the basal regions of the two species. The differing estimates of chitin content by solid-state NMR and by glucosamine analysis of hydrolysates are difficult to reconcile, but both methods

point to a higher level of chitin in *A. salix* compared to *A. fiordensis*. These two species also differ considerably in their fibrillar architecture (Kim *et al.*, 1992), making it difficult to assess the quantitative effects of individual components.

The protein component of the black coral skeleton is complex and incompletely characterized; because it is amorphous, its architecture cannot be visualized by microscopy and compared with that of chitin. However, because of its compositional dominance and by analogy with insect cuticle, many of the physical properties of black coral skeletons are likely to be related to the protein components, including the extent to which they are cross-linked to one another and to chitin (Hillerton, 1984; Hopkins and Kramer, 1992). Many of the properties of the black coral skeletons studied by Kim *et al.* (1992) (e.g. hardness, darkness, increasing hydrophobicity) are consistent with those of proteins stabilized by sclerotization (Pryor, 1962; Hillerton, 1984). The degree of sclerotization in insect cuticle, for example, has been correlated with increasing hardness or puncture-resistance (Hillerton *et al.*, 1982; Czaplá *et al.*, 1990). Other properties, such as cuticular stiffness and insolubility, have been correlated with protein characteristics (Vincent, 1980; Hackman and Goldberg, 1987).

### Sclerotization and the insect cuticle connection

Although sclerotization is apparently widespread in the animal kingdom (cf. Pryor, 1962; Brown, 1975; Waite, 1990), specific structural evidence for its occurrence is often indirect. The presence of catecholic compounds as quinone precursors (see below), proteins with a high aromatic amino acid content, or halogenated proteins may all indicate the occurrence of sclerotization (e.g. Goldberg, 1980; Tidball,

1985). As we suggested previously (Holl *et al.*, 1992), the hardening process of the skeletal structure in *A. fiordensis* may be due to the formation of aromatic cross-links between polypeptide chains. A greater quantity of diphenols in the base than in the tips, as shown by both solid state NMR and diphenol analysis of hydrolysates, is consistent with this idea. However, in the present study of *A. salix*, we found only small concentrations of diphenols in both hydrolysates and extracts and no discrete diphenol peak by NMR. These data suggest that cross-linking of proteins by quinones is less important in the stabilization of *A. salix* skeletons compared to *A. fiordensis*.

Most of the biochemical characterization of sclerotization has come from work on insect cuticle (see reviews by Pryor, 1962; Sugumaran, 1988; Andersen, 1990; Hopkins and Kramer, 1992). The most common sclerotizing agents are presumed to be quinones derived from catechols, particularly *N*-acyl derivatives of dopamine (DA), *N*-acetyldopamine (NADA) (Karlson and Sekeris, 1962), and *N*- $\beta$ -alanyldopamine (Hopkins *et al.*, 1982). Because DOPA and DA constitute the primary starting materials for the synthesis of insect melanin, their reactive amino group must be blocked by acylation to prevent internal cyclization, or metabolized by deamination. Conjugation of a diphenolic group is another way to block melanin synthesis by preventing oxidation to the quinone (Brunet, 1980; Hopkins and Kramer, 1992). The variety of potential sclerotizing agents in the Insecta may reflect, in part, the competing reactions of melanization and sclerotization. There are two reasons why melanization may not occur in black coral skeletons. Firstly, we have found little evidence of melanin in these skeletons. The black color is not extractable by solvents (Goldberg, unpublished) and is physical, apparently due to panchromatic absorption (see Fox, 1976) by the skeletal protein. Secondly, insects use low molecular weight sclerotizing agents that cross-link proteins after synthesis. Thus, substantial pools of potential cross-linking agents are often extractable using mild hydrolytic conditions (Morgan *et al.*, 1987). We have not found this in the black coral skeleton (Holl *et al.*, 1992; this study). Waite (1983, 1990) showed that DOPA is similarly difficult to extract in sclerotized proteins from several aquatic organisms, especially the Mollusca. He demonstrated that DOPA occurs as part of a protein or polypeptide, rather than as a low molecular weight precursor for sclerotization. Such a strategy should not only eliminate the possibility of intramolecular cyclization and melanin synthesis, but also should reduce the problem of

DOPA loss due to water solubility in an aquatic environment. DOPA has also been identified as a skeletal component in a hydroid (Knight, 1970), and a gorgonian (Tidball, 1982), as well as in other aquatic species (Waite, 1990). However, the inclusion of DOPA in a structural polypeptide of black coral, though suggested by our work, remains to be confirmed in coelenterates by protein isolation and amino acid analysis.

#### *Skeletal halogenation*

In some cases, elevated levels of tyrosine may suggest protein sclerotization (cf. Brown, 1975), while in others only small amounts of this amino acid may be present (Waite, 1983). Although tyrosine is not prominent in the skeletal hydrolysates of the antipathins studied here, a much larger relative amount of this amino acid remains closely associated with the skeletal chitin, suggesting a role in covalent bonding between chitin and protein. Additional tyrosine may also be halogenated or involved in stable cross-links, thereby being unaccounted for in hydrolysates analyzed for amino acid composition. Because the coral skeleton is partially or completely stabilized by sclerotization, most of the amino acids released by hydrolysis are those not involved in cross-links or protein adducts. Roche *et al.* (1963) found that mono- and diiodotyrosine in antipathin could be equal to, or greater than, the amount of non-halogenated tyrosine present. Halogenated, especially iodinated, scleroproteins are common in a large number of marine invertebrates, including sponges, annelids, molluscs, arthropods, and tunicates (Gorbman *et al.*, 1954; Roche *et al.*, 1963; Barrington and Thorpe, 1968). Iodotyrosine and chlorotyrosine have also been found in insect cuticular proteins (Wheeler, 1950; Andersen, 1972), but so far, no role for halogenated tyrosine has been suggested in insect cuticular sclerotization. In the case of marine organisms, Tong and Chaikoff (1961a,b) suggested that iodide in seawater (present at about 0.4  $\mu$ M) could be oxidized to iodine by quinones, thus spontaneously causing the formation of iodotyrosine. A similar suggestion was made by Welinder (1972) and Hunt (1976) to account for halogenated derivatives in crustacean cuticle protein and mollusc operculum, respectively. Pryor (1962) provided a more functional role for iodination by suggesting that electrophilic halogens might promote cross-link formation by generating a positive charge on phenolic hydroxyl groups. Bensusan (1966) provided experimental evidence to support this hypothesis by showing that iodination of tyrosyl or histidyl residues increases the rate of cross-link formation *in vitro*. The relative abundance

of iodine in a number of antipatharian species has been traced to tyrosyl and histidyl residues (Roche *et al.*, 1963; Goldberg, 1976). The considerably higher concentration of iodine in *A. salix* suggests the possibility of increased cross-linking in this species by an iodine-mediated mechanism. Considering the level of bromide in seawater (about 0.8 mM) and in the antipatharian skeletons, bromotyrosine (and perhaps bromohistidine) may also be present as in gorgonian coral and sponge skeletons (Roche *et al.*, 1963) but these compounds have yet to be demonstrated in black corals.

#### The possible role of histidine

Elevated levels of histidine such as those found in antipathin are occasionally reported in non-chitinous proteins (Voss-Foucart *et al.*, 1973), but more often are associated with chitin-protein complexes. Hackman (1960) reported elevated levels of histidine and aspartic acid residues in association with insect cuticle as well as cephalopod shell and pen. Because these amino acids were present after hot alkali treatment that should have removed protein, Hackman suggested they might be covalently linked with chitin, although others have disputed this finding (Attwood and Zola, 1966; Brine and Austin, 1981). In this study, relatively high levels of histidine were also strongly bound to alkali-treated chitin and were released only after acid hydrolysis. This characteristic was also shared by glycine, alanine, aspartic acid and, particularly, tyrosine. Histidine is not generally prominent in insect cuticle (Hackman and Goldberg, 1976; Richards, 1978). Nonetheless, there is evidence that it may play a prominent role in the stabilization of at least some chitin-protein complexes (Sugumaran, 1988; Hopkins and Kramer, 1992). In the tobacco hornworm, *Manduca sexta* (L.), the pupal cuticle contains about 10% histidine (Schaefer *et al.*, 1987). Kramer *et al.* (1987) found this amino acid to be the major residual species associated with cuticular chitin after deproteination with alkali. They further found a 6-fold increase in the amount of histidine relative to glucosamine in mature cuticle. In addition, work by Schaefer *et al.* (1987) and Christensen *et al.* (1991), using non-destructive solid state NMR techniques, has shown that histidyl residues in cuticular proteins are covalently bonded to catechols in tobacco hornworm cuticle, and that a histidyl-catechol adduct is probably covalently linked to chitin. This finding has parallel implications for sclerotized black coral skeleton and suggests a mechanism by which this marine chitin-protein system

may be stabilized. However, cross-linking between halogenated tyrosyl residues in proteins may also play a prominent role in stabilizing black coral skeleton, particularly among species such as *A. salix* that have a low diphenol content.

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